any amino acid (Borden, K. L. B. and Freemont, P. S., "The RING finger domain: a recent example of a sequence-structure family", *Current Opinion in Structural Biology,* **6:**395-401, 1996 and Saurin, A. J., et al., "Does this have a familiar RING?", *TIBS* **21:**208-214, (1996)). The RING finger motif is cysteine-rich and the cysteine and histidine residues function together as a unique zinc ligation system referred to as the "cross-brace' motif. Two zinc atoms are bound by this domain. BS203 (SEQ ID NO:17) contains a region that closely agrees with the RING finger motif starting at the 9th amino acid residue from the N-terminal end to the 48th amino acid residue. The RING finger motif in BS203 is the following:
CICLHVFVEPVQLPCKHNFCRGCIGEAWAKDSGLVRCPEC. The cysteine and histidine residues are underlined which match the RING finger motif above. The only difference is that the motif has two amino acid residues between the first two cysteine residues while the BS203 RING finger domain has one residue between the first two cysteines.

The RING finger family is comprised of a variety of proteins including proteins with oncogenic associations. These include the breast cancer susceptibility gene BRCA1, the RET finger protein (Rfp), the transcriptional intermediary factor (TIF1), the Cbl and Bmi-1 proto-oncoproteins and Mel18, a nuclear DNA-binding protein isolated from melanomas (Saurin, A. J., et al., "Does this have a familiar RING?", *TIBS*, **21:**208-214, (1996)). The PML protein, also a RING finger family member, is a fusion protein found in patients with acute promyelocytic leukemia (Saurin, A. J., et al., "Does this have a familiar RING?", *TIBS*, **21:**208-214, (1996)). The TRAF protein family are RING finger proteins that function in signal transduction pathways and CART1 is one that is associated with breast carcinomas and metastasis (Regnier, C.H. et al., *J. Biol. Chem.,* **270:**25715-21, (1995)). In addition, RING finger proteins have been identified which bind to steroid receptors and most likely their function is to modulate the receptors' transactivating functions. SNURF contains the RING finger motif, associates with androgen receptor, and enhances androgen receptor dependent transactivation (Moilanen, A.-M., et al., "Identification of a novel RING finger protein as a coregulator in steroid receptor-mediated gene transcription", *Molecular and Cellular Biology*, **18:**5128-5139, (1998)). Clearly, RING finger family members are strongly linked to cancer onset and proliferation.

It is of further interest that BS203 is a member of a subfamily of RING finger proteins called the tripartite subgroup which is characterized by three domains, the RING finger, the B box, and a coiled-coil domain (Saurin, A. J., et al., "Does this have a familiar RING?", *TIBS*, **21**:208-214, (1996)). The general sequence of the B box motif is C-X_2-H-X_7-C-X_7-C-X_2-C-X_5-H-X_2-H and it occurs downstream of the RING finger domain (Reddy, B. A., et al., "A novel zinc finger coiled-coil domain in a family of nuclear proteins", *TIBS*, **17**:344-45, (1992)). BS203 contains a B box downstream from its RING finger sequence beginning at the 138th amino acid residue and ending at the 170th residue. The B box sequence in BS203 is the following: CPQHNAYRLYHCEAEQVAVCQYCCYYSGAHQGH. The cysteine and histidine residues are underlined which match the B box motif above. B box domains are immediately followed by a coiled-coil domain (Reddy, B. A., et al., A novel zinc finger coiled-coil domain in a family of nuclear proteins. TIBS 17: 344-45, 1992) which is also observed for BS203. A program available on the Internet was used to analyze the BS203 sequence for a coiled-coil domain (http://www.ch.embnet.org/software/COILS_form.html). A coiled-coil domain is present immediately C-terminal to the B box domain as predicted beginning at about amino acid residue 170 and continuing to residue 250 and possibly to residue 290 of BS203 (Exhibit A). Proteins in the tripartite subgroup found to be cancer-associated are PML, Rfp, and TIF1 (Saurin, A. J., et al., "Does this have a familiar RING?", *TIBS*, **21**:208-214, (1996)).

Thus, based on the above identifying characteristics of the claimed sequences and the amendments to the claims raising the percent identity, one skilled in the art is fully enabled to identify claimed sequences and it is respectfully requested that this rejection be withdrawn.

In addition to being a RING finger family member, BS203 is highly tissue specific. Particularly, based on quantitative analysis of the occurrence of the gene product of BS203 polynucleotide in human breast tissue samples compared to human tissue samples representing the body as a whole, BS203 is approximately 22 times more abundant in breast tissue than in the rest of the body. {Data are obtained from the Lifeseq database developed by Incyte Pharmaceuticals.} The usefulness of gene products such as messenger RNA (mRNA) is well known to scientist skilled in the art of diseased tissue diagnosis. Gene products, such as messenger RNA (mRNA), that code for a particular protein that are more prevalent and highly specific to a given tissue type are extremely

useful as a marker for detection of disease in that tissue. BS203 has high tissue specificity and because of its abundance in breast tissues, is useful as a diagnostic marker for diseases of the breast.

Scientists skilled in the art of diseased tissue diagnosis agree that the appearance of a protein in a tissue or body compartment where its normal occurrence is very low or non-existent, is an indicia that the specific tissue in which the protein is normally found is in a diseased state. There are three main conditions that cause a tissue-specific protein to exist outside its specific host tissue: (1.) massive trauma, most commonly a blunt force to an organ that causes disruption of the cells; (2.) ischemia, most commonly a significant reduction or cessation of blood-flow to or through an organ that causes cell death; and (3.) hypertrophic proliferation (enlargement of an organ due to an increased size of constituent cells leading to cellular multiplication), most commonly cancer. It is this third category for which the current invention is most useful. The increase in cell number, as is understood by those skilled in the art of disease tissue diagnosis, causes an alteration to the tissue-specific protein resulting in significant amounts of that protein escaping from its specific tissue and infiltrating other non-specific tissue. Thus, absent massive trauma or ischemia, detection of a tissue-specific protein in areas of the body other than host tissue indicates that the precise disease is hypertrophic proliferation of that tissue, the most serious form being cancer.

There are many examples of the diagnostic use of tissue-specific protein markers to detect cancer. For instance, the appearance of prostate specific antigen (PSA) in seminal plasma is normal, but its detection in blood is indicative of prostate cancer. Further, the appearance of PSA messenger RNA (mRNA) in blood is indicative of prostate cancer. Likewise, the appearance of carcinoembryonic antigen (CEA) in colon and stool is normal, but its detection in blood at elevated levels is indicative of colorectal cancer. The attached Exhibit B illustrates the usefulness of tissue specific molecules which, upon detection in circulation, indicate proliferative disease. For example, Exhibit B states that CEA is expressed in normal adult tissue but is detected in serum in patients with colorectal and other carcinomas. (p. 67, col. 2):

> *Some years after the discovery the same research group found that CEA could be measured in serum from patients with colorectal cancer and other carcinomas....[s]era from healthy individuals and from patients with other*

diseases generally had low levels of CEA... CEA assays are now generally accepted as a useful and cost-efficient tool in monitoring colon cancer...

This journal article explains how a tissue specific molecule, expressed in the colon in normal individuals, is drained into lymph and blood vessels upon colon tumor growth (Fig. 5).

In addition, the attached Declaration of Dr. Paula Friedman further proves the importance and usefulness of tissue-specific markers, such as BS203. In her Declaration, Dr. Friedman illustrates the similarities between well-known markers CEA and PSA and the novel BS203 when analyzed using the Incyte database. As shown, the tissue specificity of BS203 closely resembles the tissue specificity of the above mentioned cancer markers. Clearly, the presence of BS203 outside of the breast illustrates cancer development of that tissue, just as the presence of CEA and PSA outside of their respective tissues indicates cancer of the colon and prostate, respectively. Thus, the above scientific facts support the utility of BS203 and illustrate that the appearance of BS203 protein or mRNA in a patient blood sample is indicative of breast disease in that patient.

Thus, the above scientific facts of RING finger family membership and tissue specificity support the utility of BS203 and illustrate that the appearance of BS203 protein or mRNA in a patient blood sample is indicative of breast disease in that patient.

The Examiner is reminded of the proper standard under the Revised Interim Utility Guidelines which specifically states that utility is acceptable if it is "believable to a person of ordinary skill in the art based on the totality of the evidence and reasoning provided". The Guidelines continue stating "[A]n assertion is credible unless (a) the logic underlying the assertion is **seriously** flawed, or (b) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion", (emphasis added). Simply put, the threshold to be met by Applicant is a **credible assertion** of utility, not the extraordinarily high threshold improperly held by the Examiner. Clearly, the appearance of a secreted BS203 gene product outside the breast tissue itself, such as in whole blood, urine, stool or serum, indicates a form of breast disease, akin to the presence of common markers such as PSA and CEA found in blood outside of their

prevalent tissue type. BS203's use in diagnostic test in order to determine whether a patient has a disease of the BS203 tract unquestionably illustrates a credible utility.

Therefore, it is requested that this rejection be withdrawn.

Claims 9, 13 – 20, and 24 are rejected by the Examiner under 35 U.S.C. §112 because the claimed invention is not supported by either a specific and substantial or credible asserted utility or well-established utility. The Applicant vigorously disagrees and asserts that for the reasons set forth above, one skilled in the art would know how to use the claimed invention.

Therefore, it is requested that this rejection be withdrawn.

Claims 9, 13 – 20, and 24 are rejected by the Examiner under 35 U.S.C. §112 because the claims contained subject matter which was not described reasonably enough to convey to one skilled in the art that the inventor(s) had possession of the claimed invention. Specifically, the Examiner states that the claims as drawn to polynucleotides having at least 70% identity with SEQ ID NOS. 1-14 are written too broadly. The Applicant has obviated this rejection by amending the claims to reflect polynucleotides having at least 90% identity with SEQ ID NOS. 1-14.

Therefore, it is requested that this rejection be withdrawn.

Claims 9, 13 – 20, and 24 are rejected by the Examiner under 35 U.S.C. §112 because there was no written description or characterization of the claimed nucleic acids provided in the specification. Applicant has obviated this rejection by including a full written description which includes the amendments the claims to reflect polynucleotides having at least 90% identity with SEQ ID NOS. 1-14.

Therefore, it is requested that this rejection be withdrawn.

Claim 24 is rejected by the Examiner under 35 U.S.C. §102(b) as being anticipated by fragments of Inoue. This rejection has been obviated by the amendment to the claim that now excludes the word "with" and replaces it with the phrase "over the entire length" of a specific sequence.

Therefore, it is requested that this rejection be withdrawn.

Claims 9,13-15, 17-20 are rejected by the Examiner under 35 U.S.C. §102(b) as being anticipated by fragments of Hillier et al. This rejection has been obviated by amendment to the claims such that they now exclude the word "with" which was replaced with the phrase "over the entire length" of a specific sequence.

Therefore, it is requested that this rejection be withdrawn.

Claim 16 is rejected by the Examiner under 35 U.S.C. 103(a) as being unpatentable over fragments of Hillier et al in view of Linksens. This rejection has been obviated by the amendment to the claim that now excludes the word "with" and replaces it with the phrase "over the entire length" of a specific sequence.

Therefore, it is requested that this rejection be withdrawn.

Claims19, 20, and 24 are rejected by the Examiner under 35 U.S.C. 103(a) as being unpatentable over fragments of Hillier et al in view of Inoue. This rejection has been obviated by amendment to the claims such that they now exclude the word "with" which was replaced with the phrase "over the entire length" of a specific sequence.

Therefore, it is requested that this rejection be withdrawn.

CONCLUSION

In view of the aforementioned amendments and remarks, the aforementioned application is in condition for allowance and Applicant requests that the Examiner withdraw all outstanding objections and rejections and to pass this application to allowance.

Respectfully submitted,

Maurice Cohen, *et al.*

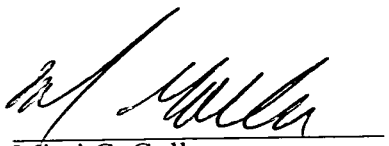

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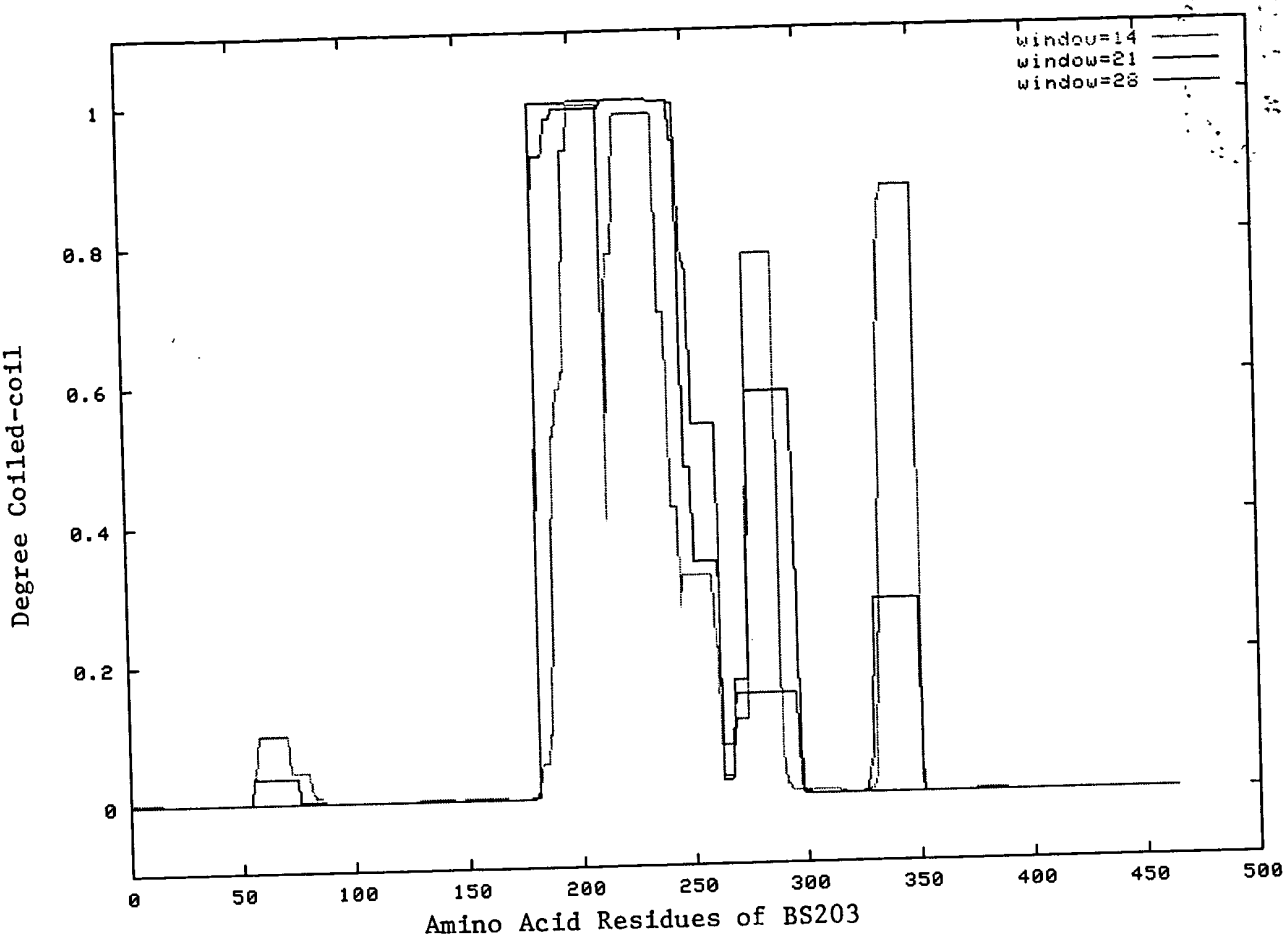
EXHIBIT A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Amino Acid Residues of BS203

seminars in CANCER BIOLOGY, Vol 9, 1999: pp. 67–81
Article No. scbi.1998.0119, available online at http://www.idealibrary.com on IDEAL

The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues

Sten Hammarström

The human CEA family has been fully characterized. It comprizes 29 genes of which 18 are expressed; 7 belonging to the CEA subgroup and 11 to the pregnancy specific glycoprotein subgroup. CEA is an important tumor marker for colorectal and some other carcinomas. The CEA subgroup members are cell membrane associated and show a complex expression pattern in normal and cancerous tissues with notably CEA showing a selective epithelial expression. Several CEA subgroup members possess cell adhesion properties and the primordial member, biliary glycoprotein, seems to function in signal transduction or regulation of signal transduction possibly in association with other CEA subfamily members. A modified ITAM / ITIM motif is identified in the cytoplasmatic domain of BGP. A role of CEA in innate immunity is envisioned.

Key words: carcinoembryonic antigen (CEA) / biliary glycoprotein (BGP) / non-specific cross-reacting antigen (NCA) / pregnancy specific glycoprotein (PSG) / CEA gene family member (CGM)

Introduction

IN THIS REVIEW I will focus on the structure, tissue distribution and possible functions of the members of the human CEA family. Some selected clinical aspects will also be discussed. The references cited in the following text are mainly from the 1980s and 1990s and for earlier literature references the reader is referred to reviews by Shively and Beatty;[1] Thompson *et al*[2] and Hammarström *et al*.[3,4]

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Milestones in carcinoembryonic antigen research

More than three decades has passed since Gold and Freedman first described the tumor associated antigen carcinoembryonic antigen (CEA) in human colon cancer tissue extracts.[5] It was hypothesized that CEA was an oncofetal antigen—expressed during fetal life, absent in the healthy adults and re-expressed in cancer. We now know that this concept does not apply to CEA. CEA is actually also expressed in normal adult tissue (see below).

Some years after the discovery the same research group found that CEA could be measured in serum from patients with colorectal and other carcinomas using a sensitive radioimmunoassay.[6] Sera from healthy individuals and from patients with other diseases generally had low levels of CEA. This created a burst of interest in this tumor marker and CEA assays were applied to a number of clinical questions related to diagnosis, staging, monitoring and prognosis of carcinomas. Since then clinical CEA assays have had their ups and downs. However, CEA assays are now generally accepted clinically as a useful and cost-efficient tool[7] in monitoring of colon cancer following surgery.

A major task in the early days was the purification and characterization of the CEA molecule. Since CEA is a complex, highly glycosylated macromolecule, this was not an easy task. Several groups made important contributions and CEA could be defined as a glycoprotein containing approximately 50% carbohydrate with a molecular weight of approximately 200 kDa.[8–12] A standard CEA preparation was developed.

Another major step in the CEA field was the discovery of CEA-cross-reactive antigens in normal human tissue including blood. Obviously the interference of these antigens in clinical CEA assays can be substantial if the antibodies used in the CEA assays

are cross-reactive. The first related antigen to be discovered was non-specific cross-reacting antigen (NCA) independently described by von Kleist *et al*[13] and by Mach and Pusztaszeri.[14] The next to follow was biliary glycoprotein (BGP) discovered by Svenberg in our group.[15,16]

In the late 1970s and early 1980s CEA became a favored target antigen for radioimmunolocalization of colorectal and other tumors of epithelial origin and with the emergence of the hybridoma technology to produce monoclonal antibodies these studies were further refined. More recently, anti CEA antibodies and derivatives have also used in experimental- and clinical-radioimmunotheraphy. Important contributors in these areas were the groups of Goldenberg, Mach and Begent (refs 17–21 and references therein). CEA has proven to be a suitable target antigen for the detection of primary and metastatic colorectal and some other carcinomas and is currently being explored as a possible target for antibody-mediated therapy.

As a consequence of the development of molecular cloning techniques in the late 1980s the entire field developed very fast and CEA has 'transformed' into an entire family of related molecules. Molecular cloning of cDNA for CEA was achieved in 1987 by four independent research groups (ref 22 and others*) followed by cDNA for NCA (ref 23 and two other groups) and BGP.[24,25] Subsequently cDNA for four additional CEA-related molecules, termed *C*EA *G*ene Family *M*ember 1, 2, 6 and 7 (CGM1, CGM2, CGM6 and CGM7) were identified.[26–29] A surprising discovery was that human pregnancy-specific β1 glycoprotein (PSG), a glycoprotein produced by placental syncytiotrophoblasts and secreted into maternal circulation[30] was indeed related to CEA.[31]

The CEA gene family

Altogether 29 different genes/pseudogenes have now been identified in the human CEA gene family.[32] Nucleotide sequence comparison between the different genes shows that the genes can be divided into three subgroups: the CEA subgroup containing 12 members; the PSG subgroup containing 11 members and the third subgroup containing six members. In the CEA subgroup seven of the 12 genes [i.e. CEA, NCA (= NCA50/90), BGP, CGM1, CGM2, CGM6 (= NCA95) and CGM7] are expressed while CGM8, CGM9, CGM10, CGM11 and CGM12 are pseudogenes. In the PSG subgroup all 11 genes may be expressed. However, for PSG7, PSG8 and PSG12 allelic variants with stop codons in the N-domain exon exist.[33] Some individuals may thus not be able to express all 11 PSGs. All six members in the third family, i.e. CGM13 to CGM18, are pseudogenes.[33] The members of the CEA gene family are clustered on chromosome 19q13.2;[34] more precisely to mid q13.2 between CY2A and D19S15[35,36] within a region of 1.8 Mb. The genome organization of the CEA family is shown in Figure 1. As can be seen the PSG and third family genes are located telomerically to the CEA subgroup genes. The genes are organized in two clusters of 250 and 850 kb separated by a region of approximately 700 kb containing several unrelated genes. Interestingly, the third subgroup genes are interspersed between the PSG genes.

Analysis of the amino acid sequence of CEA[37] and of the other members of the CEA family revealed that they belong to the immunoglobulin superfamily. Two types of immunoglobulin domains are seen: a N-terminal domain of 108 amino acids homologous to the Ig variable domain (IgV-like) and between zero and six domains homologous to the Ig constant domain of the C2 set (IgC2-like).[38] The IgC2 domains may either be of type A containing 93 amino acids or of type B containing 85 amino acids. A signal peptide of 34 amino acids (L) precedes the N-domain. This peptide is cleaved off from the mature protein following transport to the cell surface. The molecules of the CEA- and PSG-subgroups differ from each other at the C-terminal end. The CEA subgroup members are attached to the cell surface membrane while the PSGs are secreted molecules. Among the CEA subgroup members there are two types of membrane attachments. BGP, CGM1 and CGM7 contain a hydrophobic transmembrane domain (TM) followed by either a long or a short cytoplasmatic domain (CYT). CEA, NCA, CGM2 and CGM6 are attached to the cell membrane via a glycosyl phosphatidyl inositol moiety (M). The short hydrophilic tail (T) of the PSGs, of which there are four different types,[33] allows these molecules to be exported from secretory vesicles in syncytiotrophoblasts to the maternal circulation.[39] The domain formula for CEA is: [N-A1-B1-A2-B2-A3-B3-M]. For the largest BGP splice variant the formula is: [N-A1-B1-A2-TM-CYT] and for most PSG splice variants the formula is: [N-A1-A2-B2-T]. Figure 2 shows cartoons of the expressed members of the CEA sub-

* *Only one reference has been shown, however, several groups may have made the same discovery at approximately the same time.*

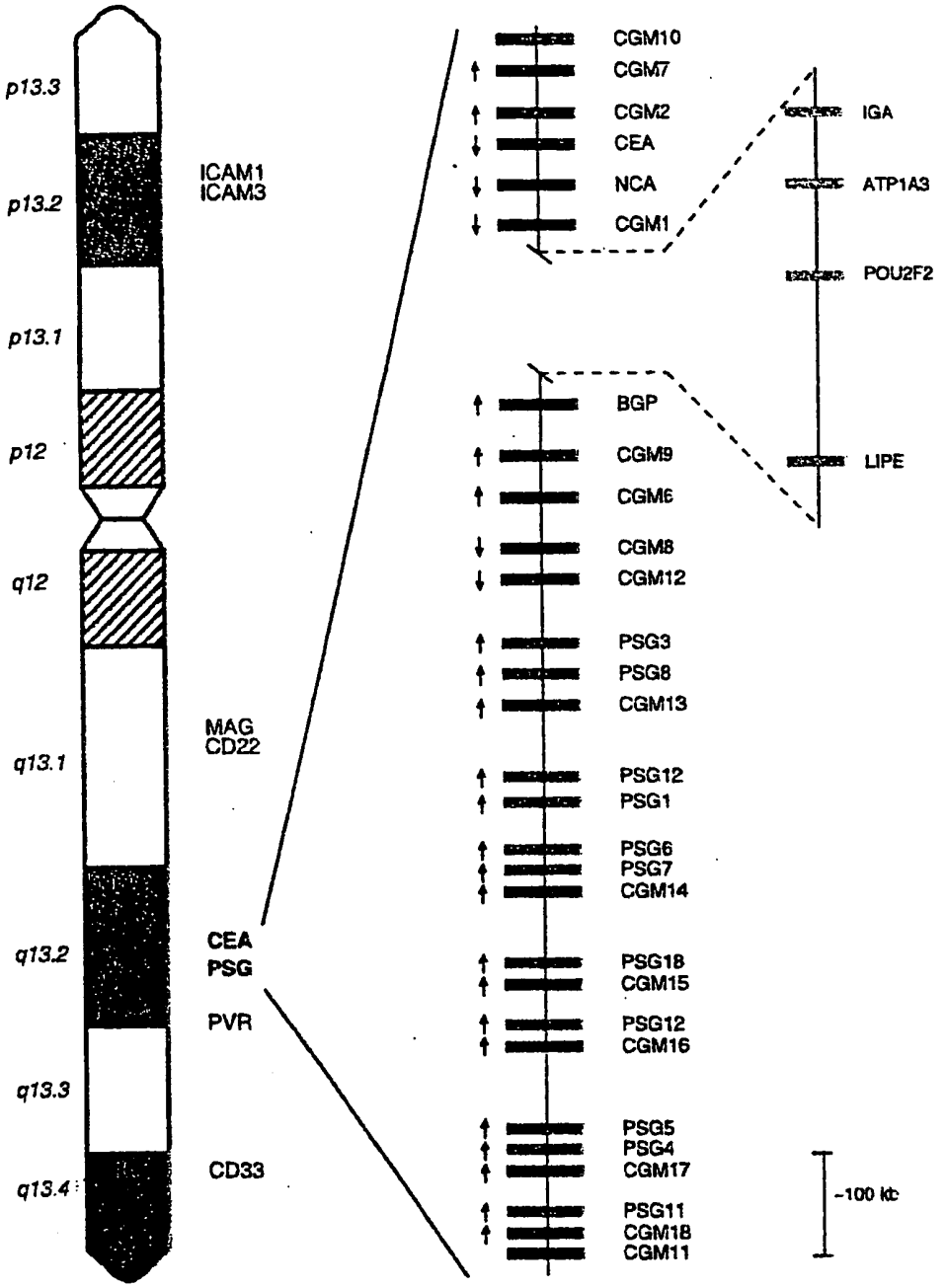

Figure 1. Genome organization of the CEA gene family. The CEA family has been localized to the q13.2 region indicated in the chromosome idiogram. Locations of the 29 individual members of the family are indicated in the expansion to the right of the chromosome. Genes are depicted as boxes of arbitrarily uniform size. Arrows indicate the direction of transcription. Boxes in the inset at far right indicate the locations of four non-CEA genes mapped within the CEA region. Other members of the immunoglobulin superfamily mapped to chromosome 19 are shown in their relative locations along the chromosome.

group. BGP, CGM1 and the PSGs occur in alternatively spliced forms. For BGP seven different forms have been described; in all forms the N-domain is retained (Figure 2). Perhaps the most important difference between the BGP splice variants is the two types of cytoplasmatic domain that exist. The longer cytoplasmatic form contains two tyrosine residues which may be phosphorylated. The tyrosines are part of modified immunoreceptor tyrosine based activation/inhibition motifs (ITAM/ITIM motifs) and may therefore participate in signaling events (ref 40 and below). Similarly, CGM1 has a long and a short cytoplasmatic domain.[41] As mentioned earlier there are four different types of short hydrophilic PSG tails. In addition we have found five different combinations of N-, A- and B-domains within the PSG group.[42] Moreover, we recently identified a second form of CGM2. It lacked the A2-domain (Figure 2; Zhou and Hammarström, unpublished results, 1998).

Two features seem to be characteristic for all expressed members of the CEA family: (i) they contain a single IgV-like N-domain, which lacks the intrachain disulfide linkage—the latter being replaced by a salt bridge. This type of IgV-domain is, however, also seen in a few other immunoglobulin superfamily members notably in CD2 and CD8; and (ii) the

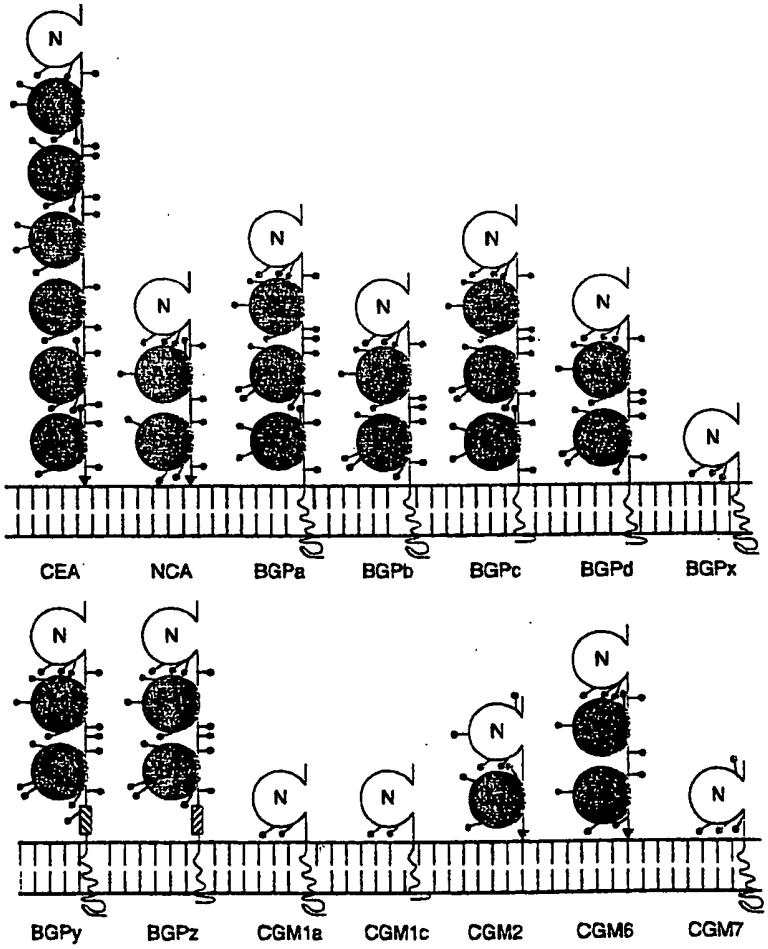

Figure 2. Models of the molecules in the CEA subgroup based on their cDNA structure. The IgV-like N-domains are unshaded and the IgC-like domains (A and B) are shaded. The non-Ig domains in BGPy and BGPz are shown as striped boxes. The GPI-linkage to the cell membrane is shown by an arrowhead. For BGP and CGM1, two forms of cytoplasmic domains (long and short) derived by alternative mRNA splicing exist. Potential glycosylation sites are shown as lollipops. Five of the CEA subfamily members have been named in the CD system; BGP is CD66a, CGM6 is CD66b, NCA is CD66c, CGM1 is CD66d and CEA is CD66e.

molecules are extensively glycosylated on aspargine residues, mostly (or exclusively) by multiantennary complex type carbohydrate chains.[43,44] The carbohydrate content may constitute up to 50% of the total mass.

Recently the structure at low resolution of the seven domains in CEA cleaved from its membrane anchor was determined by X-ray and neutron scattering.[45] CEA was found to be a monomer with a molecular mass of 150,000 with the dimensions of 20 × 8 nm (length × width) implying extended carbohydrate structures. An automated curve fitting procedure gave a family of zig-zag models for CEA. Thus CEA may be seen as a 'bottle-brush' with the immunoglobulin domains tilted at an 160° angle against each other along the long axis. Each Ig-like domain in CEA forms roughly a cylindrical shape. It is made up of two β-sheets [β-strands DEBA and β-strands GFC(C'C")] in a structure known as a β-barrel. The protein face of the GFCC' β-sheet in neighboring domains lie on alternative sides of the CEA structure. Interestingly, the protein face of the AGFCC'C" β-sheet in the N-domain and of the GFCC' β-sheet of A- and B-domains are free of steric hindrance from extended carbohydrate structures. The authors furthermore showed that this applies to several other CEA family members indicating that this face of the Ig-like domains in the molecules probably is involved in protein–protein interactions including adhesion[46] and antibody binding.[47]

Expression of CEA family members in normal human tissues

The promotor regions of the CEA family members lack the classical TATA and CCAAT elements and contain features both of constitutively active housekeeping genes like G/C rich regions, SP1 sites and of differentially or developmentally regulated genes.[22,48–50]

Until recently it has not been possible to determine the tissue expression of these molecules with certainty. This was mainly due to incomplete knowledge about the family members and their similarity to each other which has prohibited the development of specific riboprobes and specific monoclonal antibodies (mAbs). Now, however, the situation is different. Specific mAbs are available for CEA, NCA, BGP, CGM2 and for the PSGs as a group (all 11 PSGs are closely similar to each other). Similarly, specific PCR primers and riboprobes have been constructed.

Fairly extensive data on the tissue distribution are available for CEA, BGP and PSG, while only limited information is available for NCA, CGM2 and CGM6 and little is known about the tissue distribution of CGM1 and CGM7. BGP has the broadest distribution in normal tissues being expressed in a number different epithelia [esophagus (glandular epithelial cells); stomach (pyloric mucous cells, Brunner's gland cells); duodenum/jejunum/ileum (epithelial cells); colon (columnar epithelial cells, caveolated cells); pancreas (epithelial cells of the duct); liver (bile canaliculi, bile duct epithelial cells), gall bladder (epithelial cells); kidney (epithelial cells of proximal tubules); urinary bladder (transitional epithelial cells); prostate (epithelial cells); cervix (squamous epithelial cells); endometrium (glandular epithelial cells)], in sweat- and sebaceous-glands, in granulocytes and lymphocytes and perhaps also in endothelial cells in some organs.[4,51,52]

NCA has probably also a fairly broad tissue distribution being present in epithelial cells in different organs and in granulocytes and monocytes.[4,53,54]

In contrast, CEA shows a more limited tissue expression in normal adult tissue. It is present in columnar epithelial cells and goblet cells in colon, in mucous neck cells and pyloric mucous cells in the stomach, in squamous epithelial cells of the tongue, esophagus and cervix, in secretory epithelia and duct cells of sweat glands and in epithelial cells of the prostate.[4,52,55,56] CEA expression in the above mentioned organs generally commences during the early fetal period (week 9–14) and seems to persist throughout life.[55] Interestingly, mice expressing the human CEA gene under the control of its own human promotor shows essentially the same expression pattern as CEA in humans despite the fact that no CEA homologue appear to be present in rodents.[57]

The main site for PSG production is the placenta and especially during the first trimester of pregnancy the rate of synthesis is very high.[59] PSG synthesis in placenta is limited to the syncytiotrophoblast.[59] However, PSG is not exclusively expressed in human placenta. PSG cDNA clones have been isolated from fetal liver, salivary gland, testis and myeloid cells.[2,3] Most likely the PSG levels in these organs are fairly low.

Although not much is known about the distribution of the remaining four expressed molecules it would seem that CGM2 has a similar distribution to that of CEA being expressed in certain epithelial cells notably in colon but not in granulocytes. In contrast

CGM1 and CGM6 are expressed in granulocytes but probably not in epithelial cells.

To summarize, in normal adult tissue four different patterns of cellular expression can be recognized: (1) selective epithelial (CEA and CGM2); (2) granulocytic (CGM1 and CGM6); (3) selective syncytiotrophoblastic (PSGs); and (4) broad (BGP and NCA).

Expression of CEA family members in relation to cellular differentiation

We have studied the expression in normal adult colon epithelium of CEA, BGP, NCA and CGM2 (the four expressed CEA subfamily members in this tissue[58,59]) at the mRNA and protein levels using RT-PCR, *in situ* hybridization, immunohistochemistry and immunoelectron microscopy.[51,58,60] The results are summarized in Table 1. All four mRNA species were expressed at high levels in the mature columnar epithelial cells facing the free luminal surface and in the highly differentiated columnar epithelial cells at the crypt mouth. CEA- and NCA mRNAs were also expressed in the epithelial cells of mid- and lower crypt, although at lower levels. BGP- and CGM2 mRNAs, in contrast, were not detected at the lower levels of the crypt. Another difference was that mRNAs for CEA and NCA were also expressed in goblet cells, in contrast to BGP and CGM2 mRNAs. The cell- and region-specific expression patterns of CEA-, NCA-, BGP- and CGM2 glycoproteins were in complete agreement with the findings at the mRNA level indicating that the production of these glycoproteins is essentially controlled at the transcriptional level. Maximum expression of all four molecules was attained only when the columnar cell had migrated up to the level of the free luminal surface, i.e. to the functional compartment of the colonic mucosa.

Cellular differentiation-related expression was also seen for the PSGs. Using a PSG specific mAb, BAP-3, we conclusively demonstrated that PSG is only expressed in syncytiotrophoblasts, while cytotrophoblasts and extravillous trophoblasts are negative.[39]

'Fuzzy coat' (glycocalyx) localization of CEA, NCA BGP and CGM2 in normal human colon

Studies by immunoelectron microscopy using specific mAbs for CEA, NCA, CGM2 and BGP demonstrate that all four molecules are specifically localized to the apical surface of mature enterocytes. No staining is seen at the basolateral surfaces of the enterocytes with any of the four mAbs. The structure that is specifically stained is the apical glycocalyx (= fuzzy coat)/microvillus region of the mature enterocytes. The fuzzy coat is made up of microvesicles and filaments. The microvesicles are formed by the blebbing of microvillus membrane and subsequent pinching off. This vesiculation of the microvilli is a normal process and is the most distinctive feature of the 'fuzzy coat' in human colonic epithelium.[61] Figure 3 shows the precise localization of CEA, NCA, BGP and CGM2, respectively. While the CEA-positive material is mainly seen in the fuzzy coat at the tops of the microvilli, the CGM2 positive material, in contrast, is mainly seen between the sides of the microvilli. The NCA- and BGP-positive materials are seen both between the sides and over the tops of the microvilli. Thus, even within this narrow region of the cell there appears to be a degree of compartmentalization with different distribution of the four molecules.[51,58,60] It should be noted that vesiculation of microvilli is a common response to conditions that affect the intestinal microvillus membrane and perhaps serves as a rapid mechanism for the removal of membrane active agents from the gut luminal surface. The finding that CEA in normal colon is released via CEA-

Table 1. Expression of CEA subfamily members in normal adult human colon—summary of *in situ* hybridization and immunohistochemical analysis

Colonic location	Degree of expression								
	CEA mRNA	CEA Protein	NCA mRNA	NCA Protein	BGP mRNA	BGP Protein	CGM2 mRNA	CGM2 Protein	CGM1,6,7 mRNA
Crypt lower 1/3	+	+	+	+	−	−	−	−	−
Crypt middle 1/3	+	+	+	+	−	−	−	−	−
Crypt upper 1/3	++	++	++	++	++	++	++	++	−
Free luminal surface	+++	+++	+++	+++	+++	+++	+++	+++	−

Intensity of staining was scored as negative (−); weakly positive (+); positive (++) or strongly positive (+++).

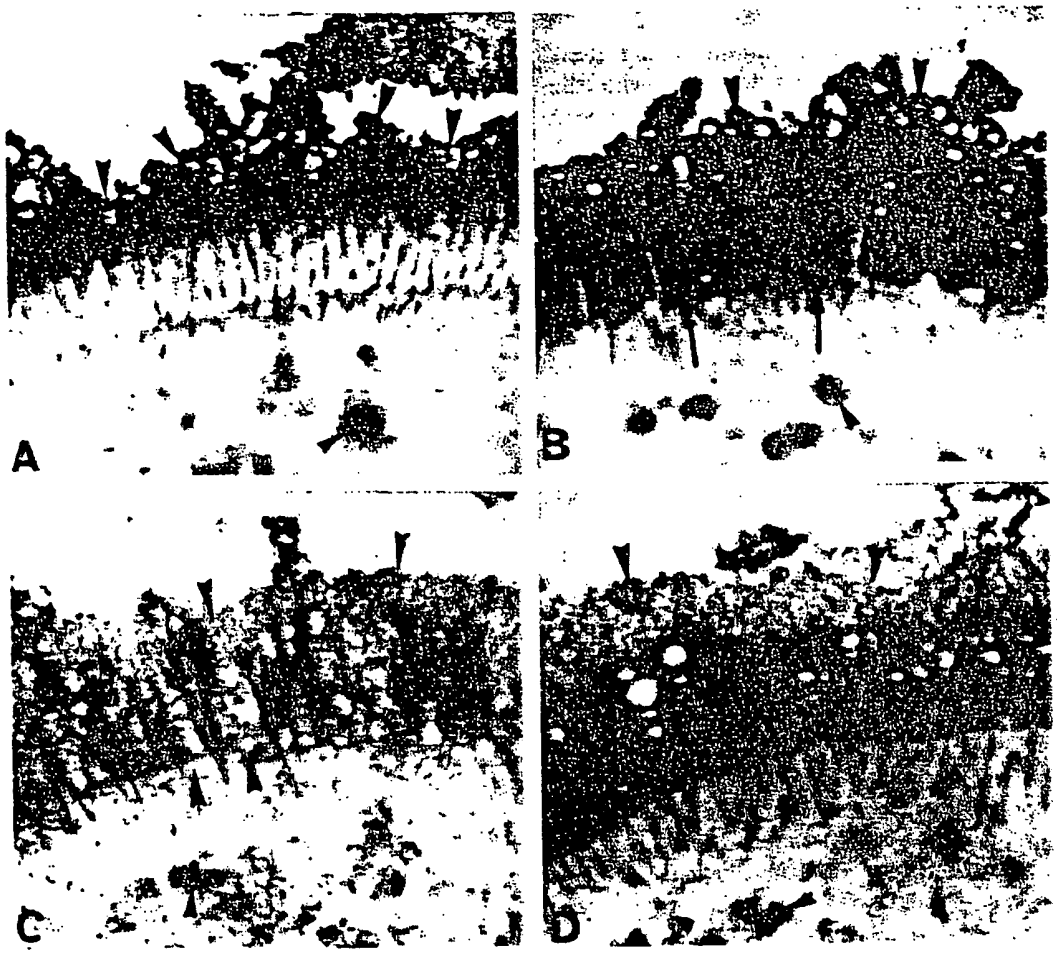

Figure 3. Immunoelectron microscopy of CEA, NCA, BGP and CGM2 in normal human colon. (A) Micrograph of apical part of a mature columnar cell. The CEA positive material is seen over the tops of the microvilli and consists of thick long filaments and membrane vesicles (thick arrowheads); ×15,000. (B) Micrograph of the apical surface of a mature columnar cell. The NCA-positive granular compact material including membrane-bound vesicles is seen between (arrows) and over the tops of the microvilli (thick arrowheads); ×15,000. (C) Micrograph of apical part of a mature columnar cell. A delicate BGP-positive material which consists of very thin loose microfilaments is located between and over the microvilli (thick arrowheads); ×16,000. (D) Micrograph of apical part of a mature columnar cell. The CGM2-positive material is present not only between the sides (arrows) but also over the top of the microvilli (thick arrowheads) and consists of fine tightly matted filaments and membrane vesicles; ×15,000. In A, B, C and D *thin arrowheads* show positively stained cytoplasmic vesicles.

coated vesicules agrees with the findings of Matsuoka *et al*[62,63] who demonstrated that more than 90% of total CEA in feces exist in a membrane bound form and that it can be released from these membranes by phosphatidylinositol-specific phospholipase C.

The production and release of CEA *in normal adult colon* is substantial. During 1 day a healthy adult evacuates approximately 50–70 mg of CEA in feces.[63] Most likely CEA is produced in even higher amounts in colon than indicated above, since Matsuoka and colleagues[62] have shown that normal colon epithelium crypts cultivated in collagen gel produce large amounts of soluble CEA, probably released through the action of some endogenous PI-PLC or PI-PLD. In the colon lumen this soluble material is rapidly broken down into smaller fragments,[62] thus even the 50–70-mg CEA produced per day is an underestimate. Moreover, direct comparison between CEA

production in normal colonic mucosa and cancerous tissues demonstrates that approximately the same amount is produced.[62,63] Taken together with the demonstration by Nap *et al*[55] that CEA is produced in fetal colon starting at the early fetal stage (week 9–13), these findings demonstrate that CEA is not a typical oncofetal antigen, rather CEA should be viewed as a normal adult tissue component with retained expression in tumors. The degree of expression is related to the state of differentiation of the normal or cancerous cell, highly differentiated cells expressing the highest levels.

Expression of CEA family members in tumors

Table 2 summarizes current information on the expression of CEA molecules in epithelial and other tumors. CEA, NCA and BGP are expressed in a number of tumors of epithelial origin such as colorectal carcinoma, lung adenocarcinoma and mucinous ovarian carcinoma and endometrial adenocarcinoma. CEA seems, however, to be somewhat more restricted in its expression pattern as compared to NCA and BGP, not being expressed in acute lymphoblastic leukemia and hepatocellular carcinoma. CGM2 is expressed in some epithelial cancers notably gastric carcinoma, and mucinous ovarian carcinoma, while the granulocyte-associated molecules CGM6 and CGM1 are not detected in any of the investigated tumors of epithelial origin. PSGs are found in hydatidiform mole and choriocarcinoma. In Tables 2 I have also indicated which molecules are thought to be up-regulated or down-regulated compared to their expression in the corresponding normal tissue. CEA is considered to be up-regulated in gastric carcinoma and possibly also in colorectal carcinoma, and the same seems to be the case for NCA. In the case of BGP the results are conflicting. BGP is considered to be down-regulated in colorectal carcinoma and in

Table 2. Expression of CEA family members in human tumors

Type of tumor	CEA*	NCA	BGP	CGM6	CGM1	CGM2	CGM7	PSG	References
Epithelial									
Colorectal carcinoma	+	+⇑	+⇓			+⇓			[27,60,64,65] [66–72]
Gastric carcinoma	+⇑	+⇑	+⇑			+⇑			[59,71,73]
Lung adenocarcinoma	+	+	+						[64,68,71,74]
squamous cell carcinoma	−		+⇑						[70,75]
Breast carcinomas	(+)	+				−			[27,67,68] [71,74]
Pancreatic carcinoma	+								[71]
Gallbladder carcinoma	+								[71]
Urinary bladder carcinoma	+								
Mucinous ovarian carcinoma	+	+	(+)	−	−	+	−		[27]
Serous ovarian carcinoma	(+)	(+)	(+)	−	−		−		[67]
Endometrial adenocarcinoma	+	+	+	−	−		−		[67]
Hepatocellular carcinoma	−		+⇓						[71,76,77]
Thyroid carcinoma	−								[71]
Nasopharyngeal carcinoma	−								[71]
Other									
Malignant mesothelioma	−								[78]
Small cell lung carcinoma	+		−						[64,75]
Acute lymphoblastic leukemia	−	+	(+)		−		−		[79]
Melanoma	−								[71]
Different sarcoma	−								[71]
Hydatidiform mole								+	[80]
Choriocarcinoma								+	[80]

Notes: +, More than 50% of individual samples were positive; (+), 10–50% of individual samples were positive; ⇑ or ⇓, indicate whether the molecule is considered to be up- or down-regulated in comparison to the corresponding normal tissue.

*Older data on CEA expression in epithelial tumors, including colon-, breast-, lung- and ovarian carcinoma, are reviewed in Shively and Beatty (1985).[1]

hepatocellular carcinoma but up-regulated in gastric carcinoma and squamous cell carcinoma of the lung. Likewise, for CGM2 the results points in both directions; down-regulation for colorectal carcinoma and up-regulation for gastric carcinoma.

An aspect, that generally is overlooked when the levels of expression of CEA family members in normal and cancer tissues are compared is the state of differentiation of the normal cells. In many cases the conclusion that a particular antigen is, for example, down-regulated in tumors will depend on whether the comparison was made against poorly or fully differentiated normal cells. Moreover the normal adjacent tissue sample usually contains several different types of cells, which may or may not express the antigen. The studies on the expression of CEA subfamily members in normal colon mucosa mentioned above illustrate the difficulties inherent to such comparisons. If compared on a cell to cell basis BGP may be considered to be up-regulated in colorectal carcinoma if compared against normal immature enterocytes and down-regulated if compared to normal mature enterocytes. These results have therefore to interpreted with great caution.

The question of whether the presence of CEA in pancreatic carcinoma and breast carcinoma (Table 2), is an example of ectopic expression can not be settled at the present time. It is quite possible that CEA is produced by a few normal cells in these organs but that the sensitivity of the methods used has precluded their detection in normal tissue.

Contrary to the strict apical localization of CEA subfamily members in normal colon epithelial cells, these molecules can be expressed over the entire cell surface of colonic adenocarcinoma cells, in intraglandular lumina and even in intracellular lumina. Figure 4 shows that CEA and NCA are expressed on the luminal surfaces of tumor cells in colonic adenocarcinoma facing intraglandular lumina. Note also that tumor cells in the inner portion of tumor gland express the antigens on almost the entire surface. Evidence for NCA synthesis can also be seen.

Is there a difference between tumor CEA and normal CEA? On the genetic level there appears to be no difference and in a classical study by Fritsche and Mach[81] it was demonstrated that normal colon CEA was indistinguishable from tumor CEA by several immunological, physicochemical and chemical criteria. However, the possibility that subtle post-translational modifications, such as trimming of the C-terminus after release from the membrane by endogenous PI-PLC, PI-PLD or modifications in the carbohydrate chains, might create differences between tumor CEA and normal CEA can still not be excluded. Another source of heterogeneity may be that CEA from different organs may display different post-translational modifications. The same type of reasoning applies to other CEA family members.

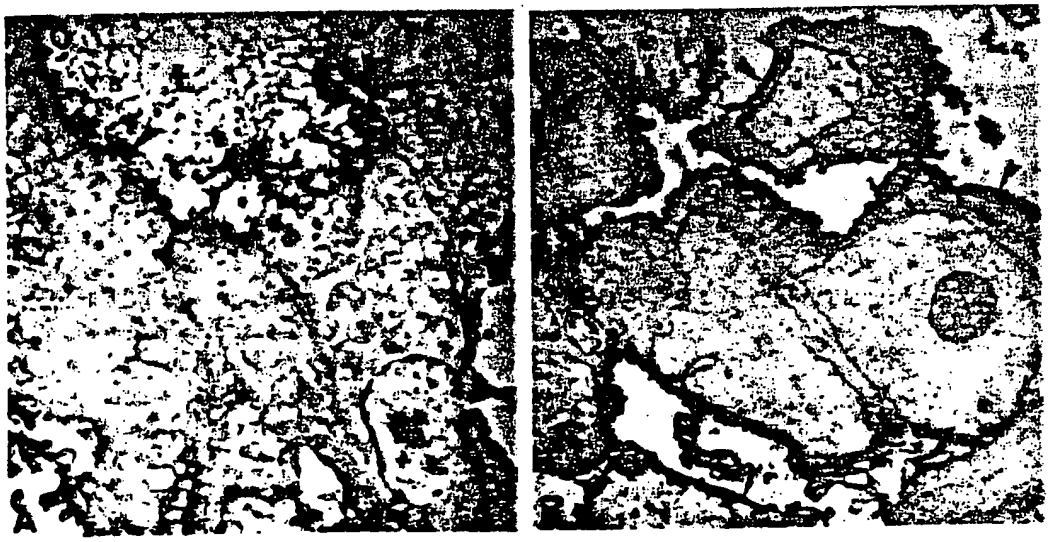

Figure 4. Immunoelectron microscopy of CEA in colon cancer. (A) Cells facing lumen (L) have a thin layer of the CEA-positive material on their luminal surface (arrowheads); ×8000. (B) Neoplastic cells of the inner portion of tumor gland show CEA on almost the entire cell surface (arrowheads); ×9000.

CEA family members as tumor markers

As mentioned above CEA is one of the most extensively used clinical tumor markers. The main reasons why CEA is useful as a serum tumor marker for colorectal and some other cancers are probably because CEA is a stable molecule, has a fairly restricted expression in normal adult tissue and is expressed at high levels in positive tumors. The bulk of the CEA in a healthy individual is produced in colon. There, it is released from the apical surface of mature columnar cells into the gut lumen and disappears with the feces (Figure 5). Thus, only very low levels are normally seen in the blood from healthy individuals. In colon cancer the malignant cells have no basal lamina and are multiplying in the tissue. Moreover, the tumor cells have lost their polarity and CEA is distributed around the cell surface. It is known that components from the plasma membrane are continually exfoliated from the surface as plasma membrane-derived vesicles,[82] which through draining lymph and blood vessels can end up in the blood. As the tumor size increases more CEA will accumulate in the blood (Figure 5).

BGP and NCA have a broader normal tissue distribution and are in addition produced by different types of white blood cells including granulocytes. The BGP and NCA levels in the blood of normal individuals are comparatively high (0.5 ± 0.3 mg/l for BGP and approx. 0.05 mg/l for NCA[83,84] as compared to < 2 μg/l for CEA). For these reasons it is unlikely that they will be more useful than CEA as clinical serum tumor markers. In the case of CGM2, however, the situation may be different. A two-site monoclonal immunoassay for CGM2 should be set up and tested on a clinical material.

The main use of serum CEA determinations as a tumor marker is in the post-surgical surveillance of colon cancer. Increased CEA levels was the first indicator of recurrent disease in 81%[85] and 89%[86] of patients, respectively. Thus, monitoring of CEA levels after treatment gives lead time and allows for second-look surgery or other treatment modalities. It has recently been shown that CEA measurement is the most cost-effective test in detecting potentially curable recurrent disease.[7] Serum CEA levels can also be used as a prognostic indicator. A high preoperative CEA level in colon cancer is associated with poor 5-years survival, while a low preoperative level is associated with good survival. On the other hand, serum CEA tests have little value for screening purposes since the number of false positive tests is too high. The test can, however, be used as a diagnostic adjunct. Very high levels are highly indicative of liver metastases.

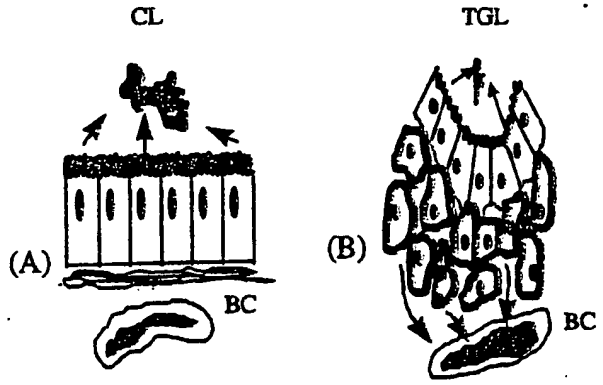

Figure 5. Major pathways of CEA excretion in normal colon and colon cancer. (A) In normal colon, polarized columnar epithelial cells express exclusively CEA on the apical surface and release it into the colon lumen (CL). CEA has no access to blood capillaries (BC). (B) In colon cancer, the epithelial cells facing 'blind' tumor gland lumens (TGL) are partly polarized and release CEA into the lumens. In contrast, the neoplastic cells located deep inside tumor glands are unpolarized and express CEA on the whole cell surface. As a result exfoliated CEA has a free access to blood- or lymphatic vessels through the intercellular spaces.

Biological functions of CEA family molecules

Cell adhesion

In vitro studies with tumor cell lines have convincingly demonstrated that several CEA subfamily members, notably BGP, CEA and NCA, can act as homophilic and heterophilic cell adhesion molecules when expressed on the tumor cell surface.[46,87,88] Intercellular adhesion is also obtained with cells containing the rat homologue to BGP (C-CAM).[89] It has also been demonstrated that the N-domain is directly involved in the cell adhesion phenomena.[40,89] Interestingly, however, it did not matter if C-CAM (= rat BGP) contained a long or a short cytoplasmatic domain.[90,91] The ITAM/ITIM motif present in the long form was therefore not important for the binding phenomena. CGM2 was not able to mediate cell adhesion.[27,59] The latter finding may actually be explained by the fact that the AGFCC'C'' β-sheet in the N-domain of CGM2 is sterically hindered by two carbohydrate substitutions (Figure 2)

The relevance of the experiments mentioned above for the situation *in vivo* must, however, be discussed. Tumor cell suspensions are used in the cell aggregation assays. On these cells, the interacting molecules are distributed over the entire cell surface. Moreover, it is known that single chain molecules belonging to the immunoglobulin superfamily are able to bind to each other forming homo- and heterodimers and higher order complexes. Thus, it is perhaps not surprising that tumor cells containing a high density of molecules such as CEA are able to aggregate in a CEA specific manner. Two questions must be posed (1) is this CEA subfamily directed cell–cell interaction important for tumors growing *in vivo*; and (2) does it occur in normal physiology when, in most cases, the cells are polarized and express the molecules only on the apical surface?

In the tumor situation it is possible that CEA family molecules play some role as a contact mediating device when tumor cells are moving to new sites. On the other hand, in tumor masses of colo-rectal carcinoma most CEA and NCA is expressed on the apical surface of tumor cells facing intraglandular lumina and not on the cell membranes between tightly binding adjacent tumor cells. The tendency of colon tumor cells to develop an apical surface is also seen when tumor cell lines, such as HT-29, are grown *in vitro*, since the cells form intracellular lumina in which CEA is deposited (Baranov and Hammarström, unpublished results). In normal physiology, it seems unlikely that CEA, NCA or BGP are involved in intercellular adhesion because of their apical localization on polarized cells, although there may be some exceptions since rat BGP (C-CAM) has been detected in intercellular contact areas in stratified epithelial cells.[92] There is no evidence indicating that the granulocyte associated CEA family molecules mediate intercellular adhesion.

In humans eight of the eleven PSGs contain the amino acid sequence arginine–glycine–aspartic acid (RGD) at a conserved and exposed site in the N-domain. This three-peptide sequence has been shown to be a recognition signal between extracellular matrix proteins and certain integrins. Thus, it was hypothesized that the PSGs act as inhibitors of cell–matrix interactions. However, our recent cDNA cloning studies of PSGs from baboon demonstrate that baboon has almost as many PSG as humans but only one of them contains the RGD sequence (Zhou and Hammarström, unpublished results, 1998). Since human and baboon are closely related species it seems unlikely that this sequence is of direct importance for the function(s) of the PSGs in primates. What then is the biological function of the PSGs? Obviously it should have something to do with pregnancy. Perhaps the PSGs acts systemically to attenuate the mother's immune response to the semiallogeneic fetus.

Signal transduction and regulation of signal transduction

As mentioned earlier one of the splice forms of BGP and CGM1, respectively, has a long cytoplasmatic tail containing modified ITAM/ITIM motif. In rat BGP (= C-CAM) the membrane proximal tyrosine residue in this motif was found to be phosphorylated by c-src in granulocytes,[93] by lyn and hck also in granulocytes[94] and by c-src in epithelial cells.[93] Protein tyrosine kinases and protein tyrosine phosphatases are bound upon phosphorylation of ITAMs and ITIMs, respectively, leading to stimulation or termination of signaling. Thus, in granulocytes, which contain phosphorylatable BGP and CGM1, in addition to GPI-linked NCA and CGM6, specific mAbs against the CEA family molecules stimulate N-formyl-MLF mediated induction of respiratory burst and β_2-integrin activation.[95,96] It seems likely that the CEA family molecules on granulocytes and perhaps also on epithelial cells such as colon enterocytes are part of a molecular receptor complexes on the cell surface similar to the CD3/TCR complex on T cells. The composition and natural ligand for these hypothetical complexes is presently unknown. The physiological receptor molecule does not necessary have to belong to the CEA family. T-cells for example can be stimulated both by antigen reacting with the TCR and by anti CD3 mAbs. It is interesting to note that BGP shares many molecular characteristics, including phosphorylable tyrosine residues in the intracellular part, with a recently discovered family of signal-regulatory proteins (SIRPs).[97] SIRPs have negative effects on cellular proliferation induced by insulin and growth factors. Recently, Beauchemin and co-workers[98,99] found that the long form of BGP could inhibit growth of malignant tumors. This exciting finding can be interpreted in terms of signal transduction regulation.

Innate immunity

It is a possible that CEA and NCA play a role in the innate immune defense protecting colon, and perhaps other areas like the upper alimentary tract, the urinary bladder and the skin (sweat glands) from microbial attack. For colon, in which organ the mi-

crobial load is the highest, the following arguments for a role in innate immunity can be made: (1) the molecules are located at a most strategic position in the apical glycocalyx facing the microbial environment in the gut; (2) CEA and NCA are produced also by goblet cells and released together with the mucins thus being present also in the outer mucinous layer directly on top of the apical glycocalyx; (3) both are heavily glycosylated proteins containing a number of multi-antennary carbohydrate chains of the GlcNAc-asparagine linkage type and of the high mannose type[43,44] with ability to interact with fimbriated bacteria.[100–102] Moreover the N-domains of CEA and NCA are recognized by the virulence associated Opa proteins in *Neisseria gonorrhoeae* and *Neisseria meningitidis*.[103,104] (4) The expression and probable release of the molecules can be regulated by inflammatory cytokines. Thus, in humans CEA and NCA probably bind and trap microorganisms preventing them from reaching down to the microvilli of the epithelial cells and invading the epithelial cell. The dynamics of the system would assure that new glycocalyx is constantly formed at the apical surface of mature enterocytes replacing 'old' glycocalyx with bound microorganisms. Moreover this process may be speeded up through signaling via BGP, since BGP can associate with CEA and NCA (but not CGM2) via exodomain interactions and BGP can transduce a signal through phosphorylation of its cytoplasmatic part (see above). Thus, if a bacterium has bound to any of these three molecules in the proximity of the cell surface the cell may sense it.

Future perspectives

In the near future it would seem particularly interesting to attempt to isolate and characterize the postulated molecular complexes containing BGP and other CEA family members on the surface of granulocytes and epithelial cells. Such analysis should include studies of the signal tranduction pathways utilized by these receptor complexes and a search for the natural ligand(s). Their relationship to the SIRPs would furthermore be interesting to investigate. It would also be of interest to investigate whether CGM2 could be used as a clinical tumor marker. A specific CGM2 immunoassay for serum analysis of patients with cancer should be developed and its performance, in comparison with the CEA assay, should be investigated. Finally it would be of interest to test the hypothesis that CEA and NCA play a role in innate immunity. This would require the development of an appropriate animal model.

Acknowledgements

I am grateful to Dr Vladimir Baranov for help and discussions in preparing this review and to Drs Marie-Louise Hammarström and Torgny Stigbrand for critically reviewing the text. This work was supported by grants from the Swedish Cancer Society and the Swedish Medical Research Council.

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